

RESEARCH PAPER

Cyclic AMP-dependent inotropic effects are differentially regulated by muscarinic G_i-dependent constitutive inhibition of adenylyl cyclase in failing rat ventricle

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BACKGROUND AND PURPOSE

β -Adrenoceptor (β -AR)-mediated inotropic effects are attenuated and G_i proteins are up-regulated in heart failure (HF). Muscarinic receptors constitutively inhibit cAMP formation in normal rat cardiomyocytes. We determined whether constitutive activity of muscarinic receptors to inhibit adenylyl cyclase (AC) increases in HF and if so, whether it modifies the reduced β -AR- or emergent 5-HT₄-mediated cAMP-dependent inotropic effects.

EXPERIMENTAL APPROACH

Contractility and AC activity were measured and related to each other in rat ventricle with post-infarction HF and sham-operated (Sham) controls with or without blockade of muscarinic receptors by atropine and inactivation of G_i protein by pertussis toxin (PTX).

KEY RESULTS

Isoprenaline-mediated inotropic effects were attenuated and basal, isoprenaline- and forskolin-stimulated AC activity was reduced in HF compared with Sham. Atropine or PTX pretreatment increased forskolin-stimulated AC activity in HF hearts. β -AR-stimulated AC and maximal inotropic response were unaffected by atropine in Sham and HF. In HF, the potency of serotonin (5-HT) to evoke an inotropic response was increased in the presence of atropine with no change in the maximal inotropic response. Interestingly, PTX pretreatment reduced the potency of 5-HT to evoke inotropic responses while increasing the maximal inotropic response.

CONCLUSIONS AND IMPLICATIONS

Although muscarinic constitutive inhibition of AC is increased in HF, it does not contribute to the reduced β -AR-mediated inotropic effects in rat ventricle in HF. The data support the hypothesis that there are differences in the functional compartmentation of 5-HT₄ and β -AR AC signalling in myocardium during HF.

Introduction

A fundamental hallmark of the failing heart is the reduced contractile response to activation of β -adrenoceptors (β -ARs) resulting from increased sympathetic drive with accompanying desensitization of the β -ARs (Tilley and Rockman, 2006). The maximal contractile response to Ca^{2+} is similar in failing and non-failing human (Ginsburg *et al.*, 1983; Fowler *et al.*, 1986; Harding *et al.*, 1990) or rat myocardium (Anand *et al.*, 1997), indicating that decreased β -AR responsiveness does not result merely from the dysfunction of the myocardial contractile machinery. Reduced β -AR responsiveness is associated with a selective reduction of β_1 -AR density resulting in an increased β_2/β_1 -AR ratio, increased expression and activity of β -AR kinases and an increase in cardiac G_i protein levels (El-Armouche *et al.*, 2003).

The level of mRNA and protein expression of G_i is up-regulated in various models of heart failure (HF) (El-Armouche *et al.*, 2003). Allegedly, increased G_i protein levels are part of an adaptive response to ischaemia, protecting cardiomyocytes from apoptosis (DeGeorge Jr *et al.*, 2008). In addition, numerous reports propose that increased G_i levels also contribute to the reduction of β -AR-mediated contractile responsiveness observed in the failing myocardium (Janssen *et al.*, 2002; El-Armouche *et al.*, 2003; Rau *et al.*, 2003). In direct support of this hypothesis, inactivation of G_i with pertussis toxin (PTX) partially restored attenuated β -AR-mediated contractility in various models of HF, including myocardial infarction in rats (Kompa *et al.*, 1999), a spontaneously hypertensive rat model (Xiao *et al.*, 2003) and in myocytes from failing human heart (Brown and Harding, 1992).

Of the adrenoceptor subtypes, β_2 -ARs display constitutive activity that is considerably higher than β_1 -ARs (Engelhardt *et al.*, 2001). In failing rat myocytes, enhanced G_i signalling selectively inhibits the inotropic effects mediated by β_2 -ARs but not those mediated by β_1 -ARs (Xiao *et al.*, 2003), probably because the β_2 -AR but not β_1 -AR couples to both G_s and G_i (Daaka *et al.*, 1997). In support of this, PTX inactivation of G_i restored the β_2 -AR-mediated contractility (Xiao *et al.*, 2003). These studies indicate that up-regulation of G_i activity with a concomitant potentiation of β_2 -AR- G_i constitutive signalling, at least in part, contributes to the reduced total β -AR-mediated contractile responses in the failing heart.

Analogous to the β_2 -AR system, constitutive activity of muscarinic receptors exerts a mild, continuous inhibition of adenylyl cyclase (AC) activity in normal rat ventricular cardiomyocyte membranes (Ricny *et al.*, 2002), probably through M_2 receptors (Jakubik *et al.*, 1995). It is well established that the muscarinic system antagonizes the inotropic responses mediated by β -ARs (Dhein *et al.*, 2001). This indirect negative inotropic response is mediated by the M_2 receptor, the predominant muscarinic receptor subtype in the mammalian cardiac ventricle (Dhein *et al.*, 2001), through activation of PTX sensitive G_i proteins and inhibition of AC (Brodde *et al.*, 2001). Furthermore, β -AR-mediated AC activation is tonically inhibited by agonist-free ('empty') muscarinic receptors in rabbit myocardium (Akaishi *et al.*, 1997). Previously, we reported that muscarinic M_2 receptor expression is increased in the rat failing left ventricle compared with that of sham-operated rats (Hussain *et al.*, 2009). In this study, we investi-

gated whether increased G_i expression coupled with an increase in muscarinic M_2 receptors in failing heart also enhances muscarinic constitutive inhibition of AC. In addition, we determined the functional effects of muscarinic constitutive inhibition of AC upon (i) the reduced β -AR-mediated inotropic effect; and (ii) the emergent cAMP-mediated inotropic effect of 5-HT (5-hydroxytryptamine, serotonin) through 5-HT₄ receptors in the rat failing ventricle (Qvigstad *et al.*, 2005a).

Methods

The experiments and animal care were conducted in accordance with 'Regulations on Animal Experimentation' under The Norwegian Animal Welfare Act and were approved by the Norwegian Animal Research Authority. Two animals per cage were housed in a temperature-regulated room with a 12 h/12 h light/dark cycle and given access to food and water *ad libitum*.

Induction of myocardial infarction and congestive HF

Male Wistar rats (Møllegaard Breeding and Research Centre, Skensved, Denmark) weighing about 300–350 g, were intubated and ventilated with 68% N_2O , 29% O_2 and 2–3% isoflurane (Abbot Scandinavia, Solna, Sweden). As described earlier (Sjaastad *et al.*, 2000) an extensive myocardial infarction was induced by proximal ligation of the left coronary artery. Mortality rate was ~10% the first day and ~65% of surviving rats developed HF by 6 weeks post-infarction. Six weeks after infarction the rats were anaesthetized again, ventilated with isoflurane and left ventricular pressures were measured. The criterion for inclusion in the HF group was a left ventricular end-diastolic pressure ≥ 15 mmHg. Sham-operated animals (Sham) underwent the same surgical procedures, except the ligation of the coronary artery. In a subset of rats, PTX was administered at a dose of 60 $\mu\text{g}\cdot\text{kg}^{-1}$ i.p. 3 days prior to isolation of the muscles. Data from animals treated with PTX were included only if carbachol inhibition of the β -AR-mediated inotropic response was completely abolished.

Isolated papillary muscles and ventricular strips

Posterior left ventricular papillary muscles and left ventricle strips (diameter ~1.0 mm) were prepared, mounted in 31°C organ baths containing physiological salt solution with 1.8 mM Ca^{2+} , equilibrated and field-stimulated at 1 Hz (Skomedal *et al.*, 1982; 1997). Contraction-relaxation cycles were recorded and analysed as previously described (Sjaastad *et al.*, 2003; Qvigstad *et al.*, 2005b). Maximal development of force $(\text{dF}/\text{dt})_{\text{max}}$, time to peak force (TPF), time to 80% relaxation (TR80) and relaxation time (RT; $\text{RT} = \text{TR80} - \text{TPF}$) were measured. Inotropic responses were expressed as increases in $(\text{dF}/\text{dt})_{\text{max}}$ and lusitropic responses were expressed as changes in RT. The descriptive parameters at the end of the equilibration period were used as basal (control) values. Prazosin (1 μM), a blocker of α_1 -adrenoceptors was added 90 min prior to agonist stimulation in the different experiments. The 5-HT₄ selective antagonist GR113808 (1 μM) was given subsequent

to 5-HT and completely reversed the 5-HT-evoked inotropic response, confirming the effect was 5-HT₄ receptor-mediated (data not shown) as previously reported (Qvigstad *et al.*, 2005a). The agonists in the study were added to the organ bath cumulatively (concentration–response relationships) or as a bolus. Concentration–response curves were constructed by estimating centiles (EC₁₀ to EC₁₀₀) and calculating the corresponding means, and the horizontal positioning is expressed as $-\log EC_{50}$ values (Sjaastad *et al.*, 2003).

Membrane preparation and AC activity assay

Membranes were prepared as previously described (Krobert *et al.*, 2001). AC activity was measured and analysed by determining conversion of [α -³²P]-ATP to [³²P]-cAMP in membranes as previously described (Krobert *et al.*, 2001). Increases in AC activity induced by isoprenaline or forskolin (experiments performed in triplicates) are reported as % increase over basal or control (stimulated in the absence of atropine).

Experimental design to evaluate possible influences of constitutive muscarinic activity

To evaluate constitutive muscarinic receptor activity, the influence of the non-selective muscarinic inverse agonist atropine was determined on basal activation of AC and that stimulated by isoprenaline, 5-HT or forskolin and on functional effects of isoprenaline and 5-HT. The influence of prior PTX inactivation of G_i upon atropine-mediated changes in AC activity and contractility were evaluated to substantiate that the effects of atropine were mediated through constitutive muscarinic receptor activation of G_i.

Statistics

Data are expressed as mean \pm SEM. $P < 0.05$ was considered to represent statistically significant differences (Student's *t*-test and ANOVA). When appropriate, Bonferroni corrections were made to correct for multiple comparisons.

Drugs and solutions

5-HT hydrochloride, (-)isoprenaline hydrochloride, timolol maleate, prazosin hydrochloride, atropine sulphate, lidocaine (2-diethylamino-N-[2,6-dimethylphenyl]-acetamide) hydrochloride, GR113808 (1-[2-[(methylsulphonyl)-amino]ethyl]-4-piperidinyl)methyl 1-methyl-1H-indole-3-carboxylate) and L-ascorbic acid were purchased from Sigma-Aldrich (St. Louise, MO, USA). Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether: Forene) was from Abbot Scandinavia (Solna, Sweden). PTX was from Merck chemicals (Nottingham, UK). Drug and molecular target nomenclature conforms with the British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Animal characteristics

All rats in the HF group had large antero-lateral infarctions and signs of congestion, including tachypnea, pleural effusion and increased lung weight. Animals in the HF group had significantly increased left ventricular end-diastolic pressure,

Table 1

Animal and papillary muscle/ventricular strip characteristics

	Sham (n = 31)	HF (n = 81)
Body weight, g	391 \pm 6	378 \pm 3
Heart weight, g	1.48 \pm 0.07	2.60 \pm 0.05*
Heart/body weight, g·kg ⁻¹	3.79 \pm 0.17	6.89 \pm 0.14*
LVEDP, mmHg	3.4 \pm 0.3	23.3 \pm 1.2*
LVSP, mmHg	121 \pm 6	97 \pm 2*
Lung weight, g	1.54 \pm 0.04	3.89 \pm 0.12*
Basal F _{max} , mN/mm ²	5.5 \pm 0.3	5.3 \pm 0.2

Student's *t*-test, * $P < 0.05$ versus Sham.

F_{max}, maximal developed force; HF, heart failure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; Sham, sham-operated.

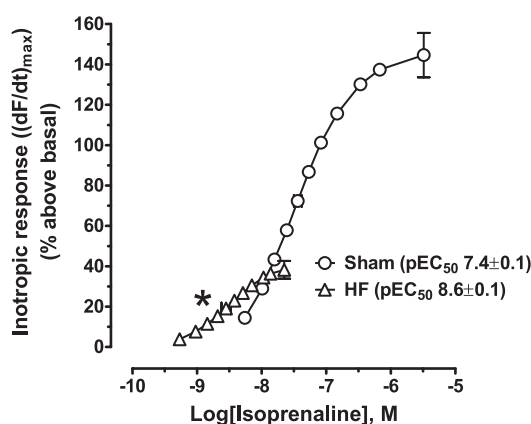


Figure 1

The inotropic response to isoprenaline is reduced, but the potency increased in heart failure (HF). Concentration–response curves for isoprenaline expressed as increase of maximum dF/dt [(dF/dt)_{max}] as % above basal in sham-operated (Sham) ($n = 14$) or HF ($n = 21$) papillary muscles. Basal F_{max} (mN/mm²) was 5.1 \pm 0.3 and 4.8 \pm 0.5 for Sham and HF respectively. Data are mean \pm SEM. * $P < 0.05$ versus Sham. CSA, cross-sectional area.

heart weight/body weight ratio, lung weight and an increased time to reach peak force in the contraction–relaxation cycle (Sjaastad *et al.*, 2003), all indicators of congestive HF. Animal characteristics and haemodynamic data at 6 weeks after infarction are given in Table 1.

The maximal inotropic response to β -AR stimulation is reduced and the potency of isoprenaline is increased in HF

Basal contractile force did not significantly differ between Sham (5.6 \pm 0.3 mN·mm⁻²) and HF (5.0 \pm 0.2 mN·mm⁻²) papillary muscles. A maximally stimulating concentration of the β -AR agonist isoprenaline (10 μ M) elicited a large sustained inotropic response [(dF/dt)_{max} 145 \pm 11% above basal, $n = 14$] in the ventricle of Sham (Figure 1). The maximal

response to isoprenaline (reached between 0.1 and 1 μM) was significantly reduced ($38 \pm 4\%$ above basal, $n = 21$, $P < 0.05$) in HF rat ventricle, approximating $\sim 26\%$ of the response in Sham (Figure 1). Interestingly, the potency of isoprenaline was increased in the HF group ($-\text{LogEC}_{50} 8.55 \pm 0.07 \text{ M}$) compared with Sham ($-\text{LogEC}_{50} 7.44 \pm 0.06 \text{ M}$, Figure 1). The potency of forskolin was also significantly increased in the HF group ($-\text{LogEC}_{50} 6.44 \pm 0.16 \text{ M}$, $n = 7$) compared with Sham ($-\text{LogEC}_{50} 5.61 \pm 0.11 \text{ M}$, $n = 8$, $P < 0.05$). Maximal forskolin-stimulated inotropic effects were also significantly reduced in HF compared with Sham (62 ± 14 and the $127 \pm 18\%$ above basal, respectively, $P < 0.05$). Although maximal forskolin-stimulated inotropic response was modestly higher than β -AR-mediated in the HF group, maximal forskolin-evoked inotropic responses were similar to those evoked by isoprenaline in the Sham.

AC activity induced by stimulation of β -ARs and forskolin is reduced in HF

Basal AC activity in HF ventricle was reduced by $\sim 16\%$ compared with Sham (26 ± 1 and $31 \pm 2 \text{ pmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, $n = 25$ and $n = 13$ for HF and Sham, respectively, $P < 0.05$). The absolute isoprenaline-stimulated AC activity above basal was decreased by $\sim 24\%$ in HF ventricle compared with Sham (Figure 2A). The absolute forskolin-stimulated AC activity above basal was decreased by $\sim 32\%$ in HF ventricle compared with Sham (Figure 2B). Isoprenaline- and forskolin-stimulated AC activities, expressed as a percentage change over basal (to account for the changes in basal AC activity), were also significantly reduced (isoprenaline: 148 ± 6 vs. $124 \pm 7\%$; 1 μM forskolin: 565 ± 19 vs. $427 \pm 12\%$; 10 μM forskolin: 1056 ± 44 vs. $836 \pm 20\%$ for Sham and HF, respectively, all $P < 0.05$).

Constitutive inhibition of AC by muscarinic receptors is increased in HF

Forskolin-stimulated AC activity was significantly increased in HF ventricle compared with Sham after inactivation of muscarinic receptors by the non-selective muscarinic inverse agonist atropine (Figure 3A), indicative of an increase in mus-

carinic constitutive activity in HF (Ricny *et al.*, 2002). The magnitude of the absolute increase in AC activation ($\sim 4 \text{ pmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$) approximated a $\sim 20\%$ increase relative to basal AC activity (Figure 3B). However, β -AR-stimulated AC activity was not increased by atropine inactivation of muscarinic receptors (Figure 3A). Muscarinic constitutive AC inhibition was abolished in HF ventricle by prior inactivation of G_i with PTX (Figure 3A,B). PTX inactivation of G_i alone significantly increased forskolin-stimulated AC activity ($\sim 20\%$) in HF ventricle (Figure 4). These data support the hypothesis that atropine's effect is through removal of the constitutive activation of G_i , because the relative increase in AC activity elicited by either atropine or PTX treatment was similar ($\sim 20\%$) (Figures 3B and 4).

Muscarinic receptor constitutive activity modulates only 5-HT₄-mediated contractility

Neither the potency (pEC_{50} of 7.5 ± 0.1 and 8.5 ± 0.1 for Sham and HF respectively) nor the efficacy (Figure 5A) of isoprenaline to induce an inotropic effect was changed in Sham or HF ventricle after inactivation of muscarinic receptor constitutive activity by the non-selective muscarinic inverse agonist atropine. Likewise, neither the potency nor efficacy of forskolin to induce an inotropic effect was increased by atropine (data not shown). However, in the presence of atropine, the potency of the 5-HT₄-stimulated inotropic effect was significantly increased by a $0.25 \pm 0.08 \text{ log unit shift}$ (Figure 5B; $\text{pEC}_{50} = 7.33 \pm 0.05$ without atropine and $\text{pEC}_{50} = 7.56 \pm 0.05$ with atropine; $P < 0.05$) without an increase in the maximal inotropic effect (Figure 5B). Prior inactivation of G_i with PTX blocked the ability of atropine to increase 5-HT potency in HF (data not shown). PTX inactivation of G_i significantly reduced the potency of the 5-HT₄-stimulated inotropic effect by $0.5 \pm 0.2 \text{ log unit}$ (Figure 5B; $\text{pEC}_{50} = 6.85 \pm 0.2$ with PTX pretreatment and $\text{pEC}_{50} = 7.35 \pm 0.05$ without PTX) while increasing the maximal 5-HT inotropic effect by $\sim 107\%$ ($\sim 36\%$ and 17% above basal, respectively, Figure 5B). Activation of AC by 5-HT was not detectable in HF ventricular membranes, but was detectable ($\sim 6\%$ increase over basal) in HF ventricles pretreated with PTX (basal AC $17.6 \pm 1.8 \text{ pmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, 5-HT-stimulated

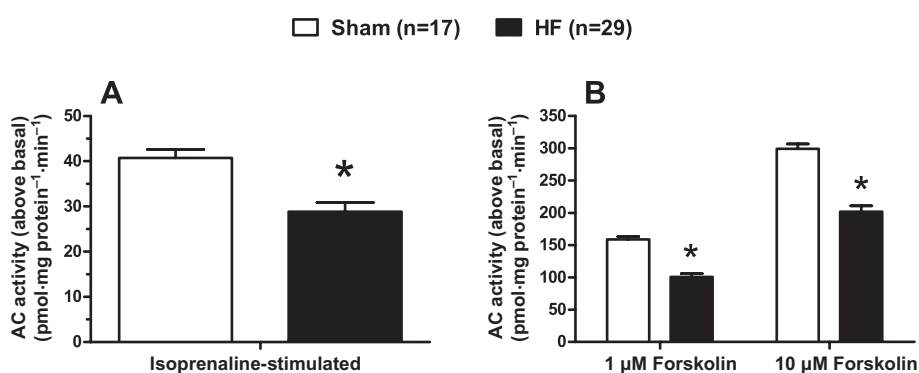


Figure 2

The stimulation of adenylyl cyclase (AC) activity by isoprenaline and forskolin is reduced in heart failure (HF). (A) Isoprenaline (10 μM) – or (B) forskolin-stimulated AC activity in membranes of left ventricle prepared from sham-operated (Sham) or HF rats. Data are mean \pm SEM. * $P < 0.05$ versus Sham.

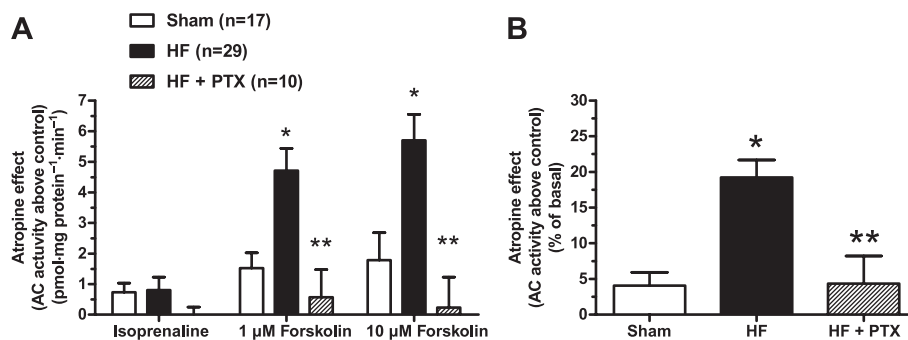


Figure 3

Atropine increases forskolin-stimulated adenylyl cyclase (AC) activity more in HF than sham-operated (Sham), and the effect of atropine is eliminated by pertussis toxin (PTX). The figure shows the effect of blocking muscarinic receptor constitutive activity with atropine (1 μM) on isoprenaline (10 μM)- or forskolin (1 and 10 μM)-activated AC activity in membranes of left ventricle from Sham, heart failure (HF) or HF rats pretreated with PTX. (A) Data shown are the mean \pm SEM of the absolute difference in AC activity induced by isoprenaline or forskolin in the presence of atropine minus that in the absence of atropine (control). (B) The combined mean of the difference scores of 1 and 10 μM forskolin-stimulated AC activity in membranes of left ventricle (same data from A) reported as a percentage of basal AC activity for each respective group. * $P < 0.05$ versus Sham only. ** $P < 0.05$ versus HF group.

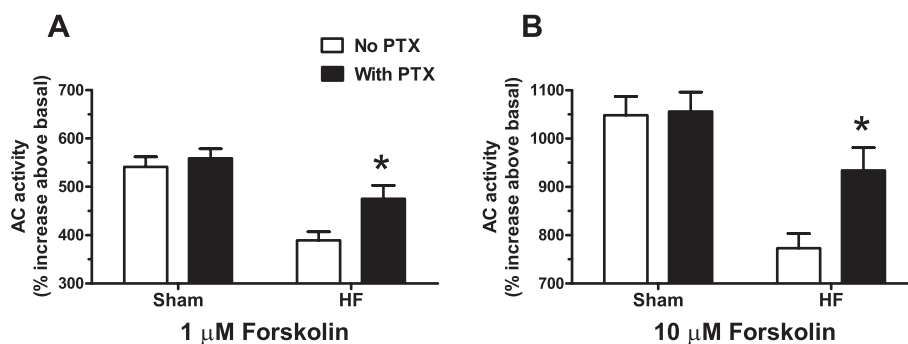


Figure 4

Pertussis toxin (PTX) pretreatment counteracts the reduction in forskolin-stimulated adenylyl cyclase (AC) activity in heart failure (HF). The figure shows the effect of pretreatment with PTX on 1 μM (A) and 10 μM (B) forskolin-stimulated AC activity in membranes of left ventricle from sham-operated (Sham) or HF rats. Data are mean \pm SEM and are reported as % increase over basal. * $P < 0.05$ versus without PTX.

18.6 ± 1.8 pmol·mg protein⁻¹·min⁻¹, $n = 8$, $P < 0.05$), consistent with the increased 5-HT₄-mediated inotropic effect.

Discussion and conclusions

Constitutive muscarinic receptor activity is enhanced in failing ventricle

The present study indicates that constitutive activity of muscarinic receptors to inhibit AC is increased in the left ventricle of rats with HF compared with Sham. Our data do not support the hypothesis that an increase of constitutive muscarinic receptor inhibition of AC contributes to the reduced β -AR-mediated inotropic effect in failing ventricle. However, muscarinic receptor constitutive inhibition of AC appears to reduce the magnitude and potency of cAMP-mediated inotropic effects evoked by stimulation of 5-HT₄ receptors in failing ventricle. Our results are consistent with

previous reports that muscarinic receptors constitutively inhibit AC activity in normal rat ventricle (Rigny *et al.*, 2002); and to our knowledge, these data are the first to demonstrate a functional significance of constitutive muscarinic receptor activity altering contractility in the failing heart.

Muscarinic constitutive inhibition of AC was significantly enhanced in HF ventricle (~20% increase relative to basal AC; Figure 3A,B), by a magnitude that corresponds well with the increase in M₂ receptor expression (~26%) previously reported by us (Hussain *et al.*, 2009). The absolute magnitude of muscarinic constitutive inhibition of forskolin-stimulated AC remained constant in both Sham and HF, despite a doubling in forskolin-stimulated AC activity when the concentration increased from 1–10 μM (see Figures 2 and 3). Therefore, muscarinic constitutive inhibition of AC is independent of forskolin concentration, indicating muscarinic constitutive inhibitory effects are limited to a localized subset of the AC that is accessible to forskolin.

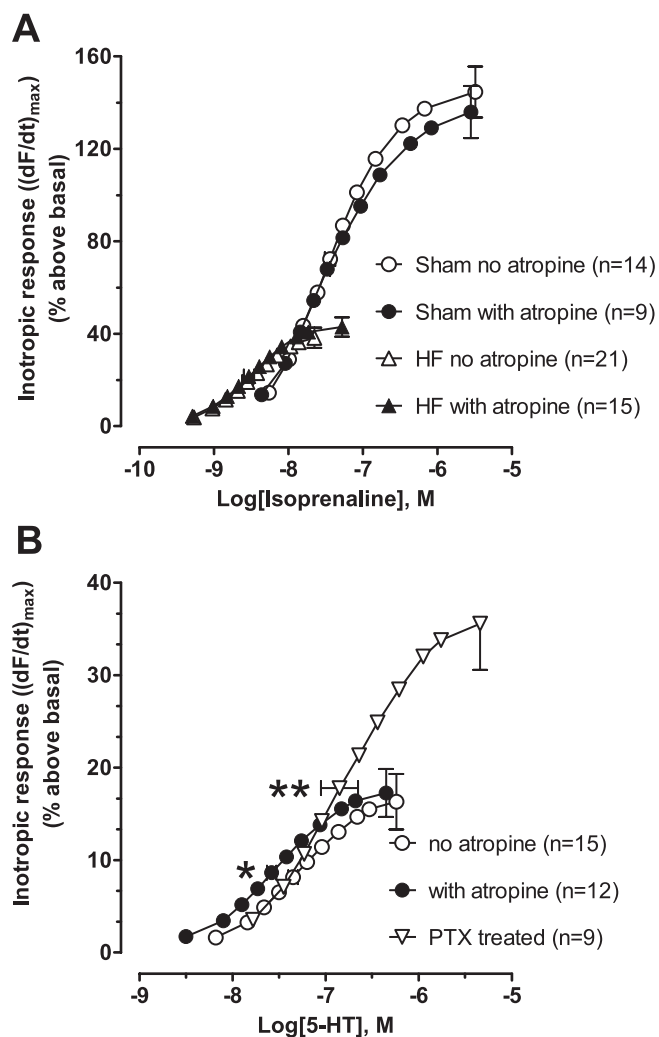


Figure 5

Atropine sensitizes heart failure (HF) ventricles to 5-hydroxytryptamine (5-HT)- but not isoprenaline-evoked inotropic effects. (A) Concentration–response curves of the inotropic response to isoprenaline in HF or sham-operated (Sham) papillary muscles in the absence or presence of 1 μ M atropine. Inotropic response is expressed as increase of maximum dF/dt [(dF/dt)_{max}] as % above basal. Basal F_{max} (mN/mm²) was 5.1 ± 0.3 and 5.9 ± 0.4 for Sham and 4.8 ± 0.5 and 5.0 ± 0.3 for HF in the absence and presence of atropine, respectively. Data are mean \pm SEM. (B) Effects of atropine (1 μ M) and pertussis toxin (PTX) on the concentration–response curve of the inotropic response to 5-HT in HF papillary muscles. The 5-HT concentration–response curve for the PTX-treated group is the combined data of muscles with and without atropine from nine rats, because atropine did not significantly alter potency in PTX-treated rats. Basal F_{max} (mN/mm²) was 5.4 ± 0.4 and 5.2 ± 0.5 in the absence and presence of atropine, respectively, and 4.9 ± 0.3 in the PTX-treated group. Data are mean \pm SEM. * $P < 0.05$ versus no atropine. ** $P < 0.05$ versus non-PTX treated.

Constitutive muscarinic receptor activity is dependent on G_i

Inactivation of G_i with PTX completely blocked the ability of atropine to increase forskolin-stimulated AC activity, indicat-

ing atropine's effect is probably due to binding at the G_i -coupled muscarinic M_2 receptor (Brodde *et al.*, 2001). Inactivation of G_i alone also increased forskolin-stimulated AC activity only in HF, not Sham (Figure 4). This finding is in accordance with previous reports indicating G_i activity is increased in the failing heart (El-Armouche *et al.*, 2003). In fact, atropine and PTX pretreatment both increased forskolin-stimulated AC activity by relatively the same magnitude (~20% increase when compared with basal and ~20% increase above basal, respectively, Figures 3B and 4). The data do not allow us to determine if the increased effect of atropine in failing heart results from the generalized increase in G_i activity only or from other mechanisms regulating AC activity. An increase in muscarinic receptor levels in failing rat ventricle (Hussain *et al.*, 2009) may also be a contributing factor. Interestingly, neither the potency nor efficacy of isoprenaline-evoked inotropic effects was enhanced by atropine (Figure 5A). This finding is consistent with the absence of atropine effects upon isoprenaline activation of AC (Figure 3A). However, β -AR-mediated activation of AC was potentiated by PTX inactivation of constitutive G_i activity in rabbit ventricular myocardium (Akaishi *et al.*, 1997), indicating the functional role of constitutive G_i activity varies among species.

Inactivation of G_i enhances the 5-HT-evoked inotropic effect and normalizes the relationship between inotropic and lusitropic concentration–response curves

Inactivation of G_i with PTX did not increase 5-HT potency (as expected if removing tonic G_i inhibition similar to atropine) but rather reduced the potency of 5-HT to elicit inotropic effects (rightward shift in EC_{50} , Figure 5B). Typically, a reduction of drug potency is indicative of a desensitized receptor system and would normally be coupled with a concomitant reduction in receptor–effector coupling (efficacy). However and perhaps the most intriguing finding of this study, the reduced 5-HT potency was coupled with the unexpected and counter-intuitive finding that 5-HT-evoked maximal inotropic effects were significantly amplified after G_i inactivation (Figure 5B).

We propose that this paradoxical effect of G_i inactivation upon the 5-HT₄-evoked inotropic effect possibly results from a restoration in the correlation between the inotropic and lusitropic concentration–response curves by PTX. The potency of 5-HT to mediate the inotropic effect is higher compared with the lusitropic effect in non-PTX-treated rats (Figure 6A). At higher 5-HT concentrations the divergence is greater, indicating 5-HT elicits a lusitropic effect well after the inotropic effect has reached maximum (~4.0 μ M and 0.4 μ M, respectively; Figure 6A). Similar results have been reported for β -AR-evoked inotropic effects in failing rat ventricle (Qvigstad *et al.*, 2005b). In contrast, the maximal inotropic and lusitropic effects were achieved over the same concentration range for β -AR stimulation in Sham (Qvigstad *et al.*, 2005b). In HF ventricle, possibly the lusitropic effect is increased relative to the inotropic component, impeding development of the maximal inotropic response.

In PTX-treated rats, the maximal inotropic and lusitropic effects were achieved over a similar concentration range of

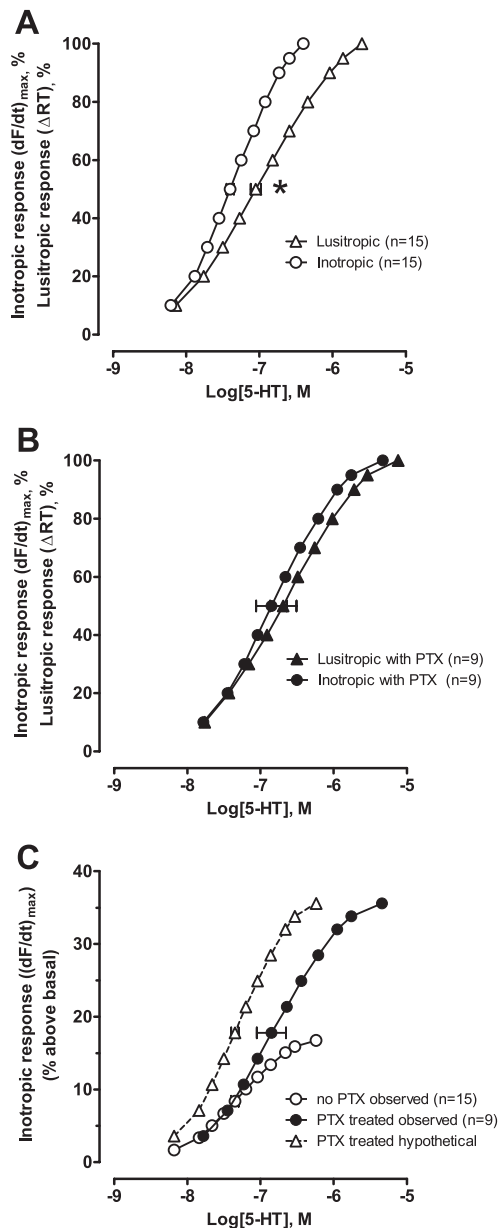


Figure 6

Concentration–response curves for functional effects of 5-hydroxytryptamine (5-HT) in heart failure (HF) papillary muscles from (A) rats without or (B) pretreated with pertussis toxin (PTX). Inotropic [increase of (dF/dt)_{max}] and lusitropic responses [reduction of relaxation time (ΔRT)] are expressed as % of maximum within each experiment. Data are mean \pm SEM. * $P < 0.05$ inotropic versus lusitropic EC_{50} in non-PTX treated rats. (C) Inotropic [increase of (dF/dt)_{max}] concentration–response curves for 5-HT in HF papillary muscles from rats pretreated with or without PTX plotted as the actual group mean (% above basal). The maximal 5-HT-evoked inotropic effect plotted was the mean from muscles with and without atropine (16.7 and 35.6% above basal without and with PTX pretreatment respectively). The hypothetical curve (open triangles) in (C) illustrates the expected concentration–response curve for 5-HT if PTX treatment did not change potency (obtained by multiplying the response at each concentration with the same factor to adjust for increased efficacy). Thus, the unexpected reduction in potency of the observed data is clearly visualized, revealing additional effects of PTX.

5-HT stimulation (Figure 6B). Therefore, inactivation of G_i normalizes the relationship between inotropic and lusitropic concentration–response curves similar to that observed with β -AR-mediated effects in Sham or normal rat ventricle. The current data do not distinguish if PTX inactivation of G_i directly enhances the 5-HT inotropic effect or modifies the interaction of the lusitropic effect upon the inotropic. However, because PTX removal of G_i inhibition did not increase the maximal 5-HT lusitropic effect (no change in relaxation time, data not shown) or the concentration–response range (see Figure 6A,B), it seems more likely that G_i inactivation directly modifies the maximal inotropic response. In support of this, PTX inactivation of G_i increased 5-HT-evoked AC activity in the current study, increased 5-HT_{4(b)}-mediated cAMP formation in HEK293 cells (Pindon *et al.*, 2002) and L-type Ca^{2+} current in rat cardiomyocytes (Pindon *et al.*, 2002), all changes supportive of an increased inotropic effect.

The 5-HT₄ receptor subtype mediating inotropic effects in failing ventricle may couple dually with G_s and G_i

The 5-HT_{4(b)} receptor subtype, that couples dually to G_s and G_i (Pindon *et al.*, 2002), may account for the sensitivity of the 5-HT inotropic response to G_i inhibition and the divergence in lusitropic and inotropic effects as discussed above. As seen in Figure 6C, the 5-HT concentration–response curves in PTX-pretreated hearts were virtually identical to those in untreated hearts at lower 5-HT concentrations, but the maximal effect was achieved at higher 5-HT concentrations. This effect of PTX is unlikely to be due to an alteration in G_s -protein activation, because PTX inactivation of G_i altered GTP γ S binding and AC activation of only the dually coupled 5-HT_{4(b)} and not the G_s -coupled 5-HT_{4(a)} subtype (Pindon *et al.*, 2002). Our data fits best with a model whereby dual coupling is dependent on agonist concentration, similar to that reported for the dopamine D₂ receptor (Chang *et al.*, 1997). Thus, the possible dual coupling of 5-HT_{4(b)} receptors to G_i at higher 5-HT concentrations may constrain the maximal 5-HT₄-evoked inotropic effect by impeding G_s activation. Removal of the G_i inhibition by PTX would allow further development of the inotropic response at the higher 5-HT concentrations (extending the concentration–response range), giving the false appearance of a reduction in 5-HT potency (EC_{50} rightward shift, Figures 5B and 6C). In comparison, if we assume dual coupling to G_s and G_i is independent of agonist concentration, after PTX inactivation of G_i , the 5-HT inotropic effect would be expected to be larger (increased G_s activation of AC) over the entire concentration range (EC_{50} remains the same) to reach the maximal inotropic response (Figure 6C, hypothetical curve). In fact, the EC_{50} of 5-HT to evoke inotropic effects in PTX-treated heart begins to approach the EC_{50} of 5-HT to activate AC at 5-HT_{4(b)} receptors expressed in HEK293 cells (pEC_{50} of 6.91 and \sim 6.7–7.2, respectively; Bruheim *et al.*, 2003), indicating PTX restores the relationship of the potency to activate AC with the functional response. Regardless of mechanism, in rat ventricles, clearly G_i plays a key role in the compartmentation of 5-HT₄-evoked AC signalling to access the contractile apparatus.

Muscarinic, β -AR and 5-HT receptor systems are localized to different functional compartments

Given the relatively large absolute muscarinic constitutive inhibition revealed by forskolin-stimulated AC (~16% of the isoprenaline-stimulated increase of AC), it is intriguing that atropine did not significantly increase isoprenaline-stimulated AC activity in HF (Figure 3A). The differential sensitivity of β -AR and 5-HT receptor systems to muscarinic constitutive activity may, in part, be due to differences in compartmentation. Head *et al.* (2005) have shown that β -ARs and muscarinic M_2 receptors with their associated signalling components (G_s and G_i respectively) vary in their subcellular distribution in adult rat cardiomyocytes. Whereas the β_1 -AR was primarily localized to low-density gradient fractions with higher levels of caveolin, M_2 receptors were only found in high-density fractions with lower caveolin levels (Steinberg, 2004; Head *et al.*, 2005). Interestingly, the cholinergic agonist carbachol promotes the translocation of M_2 receptors into the low-density fraction (Feron *et al.*, 1997) and may account for M_2 -mediated accentuated antagonism upon β_1 -AR stimulation (Iancu *et al.*, 2007; 2008). Importantly, atropine did not induce translocation of M_2 and also prevented translocation by carbachol (Feron *et al.*, 1997). These findings are highly relevant, because they provide a structural basis for the possibility that β_1 -ARs and M_2 receptors reside in different subcellular microdomains, possibly explaining why atropine did not modify β -AR-mediated inotropic responses in the current study. It is reasonable to propose that in normal rat ventricle, 5-HT₄ receptors are localized in subcellular microdomains that are functionally defined by an uncoupling of the production of cAMP from the phosphorylation of contractile proteins [analogous to prostaglandin receptors (Warrier *et al.*, 2007)] that mediate inotropic support [5-HT does not evoke inotropic effects in normal rat ventricle (Qvigstad *et al.*, 2005a)]. Perhaps, 5-HT₄ receptors are localized to the same subcellular microdomain as muscarinic M_2 receptors, and thereby are susceptible to the enhanced muscarinic constitutive inhibition of AC in HF ventricle.

In summary, these data demonstrate that muscarinic constitutive activation of AC is increased in HF rat ventricle. Removal of constitutive muscarinic inhibition of AC by atropine and PTX inactivation of G_i revealed differences in functional compartmentation of 5-HT₄ and β -AR AC signalling in HF myocardium. Additional biochemical studies coupled with confocal microscopic evidence are required to substantiate and further clarify the role of muscarinic receptors and G_i to regulate compartmentation of 5-HT₄- and β -AR-mediated inotropic and lusitropic effects in the failing ventricle.

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Conflict of interest

The authors state no conflict of interest.

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